

SOLID PHASE SEQUENCING

Cross Reference to Related Applications

5 This application claims priority to United States provisional application number 60/445,193 filed February 5, 2003; the disclosures of which are incorporated herein by reference in their entirety.

Field of the Invention

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 The present invention relates generally to methods of sequencing a polynucleotide in a sample, based on the use of terminal-phosphate-labeled nucleotides containing three or more phosphates as substrates for nucleic acid polymerases. The labels employed are enzyme-activatable and include
15 chemiluminescent, fluorescent, electrochemical and chromogenic moieties as well as mass tags.

Background of the Invention

20 Methods are known for detecting specific nucleic acids or analytes in a sample with high specificity and sensitivity. Such methods generally require first amplifying nucleic acid sequence based on the presence of a specific target sequence or analyte. Following amplification, the amplified sequences are detected and quantified. Conventional detection systems for nucleic acids include detection of fluorescent
25 labels, fluorescent enzyme-linked detection systems, antibody-mediated label detection, and detection of radioactive labels.

 One disadvantage of detection methods widely in use is the need to separate labeled starting materials from a final labeled product or by-product. Such
30 separations generally require gel electrophoresis or immobilization of a target sequence onto a membrane for detection. Moreover, there are often numerous reagents and/or incubation steps required for detection.

It has been known that DNA and RNA polymerases are able to recognize and utilize nucleosides with a modification at or in place of the gamma position of the triphosphate moiety. It is further known that the ability of various polymerases to recognize and utilize gamma-modified nucleotide triphosphates (NTP's) appears to vary depending on the moiety attached to the gamma phosphate. In general, RNA polymerases are more promiscuous than DNA polymerases.

A colorimetric assay for monitoring RNA synthesis from RNA polymerases in the presence of a gamma-phosphate modified nucleotide has been previously reported. In this prior report, RNA polymerase reactions were performed in the presence of a gamma-modified, alkaline phosphatase resistant nucleoside triphosphate, which was modified at its gamma-phosphate with a dinitrophenyl group. When RNA polymerase reactions were performed in the presence of this gamma-modified NTP as the sole nucleoside triphosphate and a homopolymeric template, it was found that RNA polymerase could recognize and utilize the modified NTP. Moreover, when the polymerase reactions were performed in the presence of an alkaline phosphatase, which digested the *p*-nitrophenyl pyrophosphate aldo-product of phosphoryl transfer to the chromogenic *p*-nitrophenylate, an increase in absorbance was reported. A disadvantage of this detection method is that the real-time colorimetric assay, performed in the presence of an alkaline phosphatase, only works with a homopolymeric template and thus can not be used for sequence analysis of a heteropolymeric template.

It would, therefore, be of benefit to provide a method for detecting RNA in the presence of a heteropolymeric template, which method would not be restricted to using a single terminal-phosphate modified nucleotide as the sole nucleotide that is substantially non-reactive to alkaline phosphatase. This would allow for a single-tube assay for real-time monitoring of RNA synthesis using hetero-polymeric templates.

It would further be of benefit to provide for similar assays for RNA wherein the identity of the label on the terminal-phosphate is varied to allow for better recognition and utilization by RNA polymerase. Furthermore, it is desired that the label on the terminal-phosphate could be varied so as to allow for chemiluminescent and fluorescent detection, analysis by mass or reduction potential, as well as for

improved colorimetric detection, wherein only simple and routine instrumentation would be required for detection.

Given that DNA polymerases are known in the art to be less promiscuous than RNA polymerases regarding recognition and utilization of terminally-modified nucleotides, wherein the identity of the moiety at the terminal position can largely affect the DNA polymerase's specificity toward the nucleotide, it would be highly desired to provide for a non-radioactive method for detecting DNA by monitoring DNA polymerase activity. Furthermore, it would be desired that the synthesis and sequence determination of DNA could be accomplished in a single-tube assay for real-time monitoring and that the label at the terminal-phosphate of nucleotide substrates could encompass chemiluminescent, fluorescent, and colorimetric detection, as well as analysis by mass or reduction potential.

Summary of the Invention

The present invention provides for a method of detecting the presence of a nucleic acid sequence including the steps of: a) conducting a nucleic acid polymerase reaction, wherein the reaction includes the reaction of a terminal-phosphate-labeled nucleotide, which reaction results in the production of labeled polyphosphate; b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable species; and c) detecting the presence of the detectable species. A definition of phosphatase in the current invention includes any enzyme which cleaves phosphate mono esters, phosphate thioesters, phosphoramidates, polyphosphates and nucleotides to release inorganic phosphate. In the context of the present invention, this enzyme does not cleave a terminally labeled nucleoside phosphate (i.e. the terminal-phosphate-labeled nucleoside polyphosphate is substantially non-reactive to phosphatase). The phosphatase definition herein provided specifically includes, but is not limited to, alkaline phosphatase (EC 3.1.3.1) and acid phosphatase (EC 3.1.3.2). The definition of a nucleotide in the current invention includes a natural or modified nucleoside phosphate.

The present invention further provides a method of sequencing a nucleic acid sequence by a) immobilizing one of the key components of the sequencing reaction,

such as polymerizing enzyme, primer, template or a complex formed by mixing 2 or more of these components, b) allowing the hybridization to proceed unless it was already done prior to step a, c) incubating in the presence of a nucleic acid polymerizing enzyme, a phosphatase and a terminal-phosphate-labeled nucleoside polyphosphate, which reaction produces labeled polyphosphate if the nucleotide present is complementary to the target sequence at the site of polymerization. The labeled polyphosphate then reacts with phosphatase or a phosphate or polyphosphate transferring enzyme to produce free label with a signal readily distinguishable from the phosphate bound dye. If the nucleotide added is not complementary to the target sequence at the site of polymerization, no polymerization takes place and no free label is produced. Thus, formation of free label identifies the base added and hence the target sequence. After sufficient time is allowed for the polymerization reaction, which may range from milliseconds to several minutes, and detecting the presence or absence of signal, solid support may be separated from solution by any of the means known in the art, including but not limited to washing, filtration, centrifugation, decantation, etc., and next nucleotides may be added in the presence of fresh polymerase (if needed) and phosphatase. It should be noted that phosphatase may be added after the polymerization has already proceeded.

According to the above description the invention provides a method of sequencing a target region of a nucleic acid template, comprising:

a) conducting a nucleic acid polymerization reaction on a solid support, by forming a reaction mixture, said reaction mixture including a nucleic acid template, a primer, a nucleic acid polymerizing enzyme, and one terminal-phosphate-labeled nucleoside polyphosphate selected from a nucleoside with a natural base or a base analog

wherein a component of said reaction mixture or a complex of two or more of said components, is immobilized on said solid support, and said component or components are selected from the group consisting of said nucleic acid template, said primer, and said nucleic acid polymerizing enzyme,
and

said reaction results in production of labeled polyphosphate if said terminal-phosphate-labeled nucleoside polyphosphate contains a base complementary to the template base at the site of polymerization;

- b) 5 subjecting said reaction mixture to a phosphatase treatment, wherein a detectable species is produced if said labeled polyphosphate is produced in step a);
- c) detecting said detectable species;
- d) continuing said polymerization reaction by adding a different terminal-phosphate-labeled nucleoside polyphosphate selected from the remaining
10 natural bases or base analogs to said reaction mixture and repeating steps b and c; and
- e) identifying said target region sequence from the identity and order of addition of terminal-phosphate labeled nucleoside polyphosphates resulting in
15 production of said detectable species.

15 The present invention further provides methods of sequencing a target using the steps described above in a continuous flow or a stop-flow system, where the immobilized material is held in place by any one of the means known in the art and different reagents and buffers are pumped in to the system at one end and exit the
20 system at the other end. Reagents and buffers may flow continuously or may be held in place for certain time to allow for the polymerization reaction and phosphatase hydrolysis to proceed.

25 The invention further provides for a method of detecting the presence of a DNA sequence including the steps of: a) conducting a DNA polymerase reaction in the presence of a terminal-phosphate-labeled nucleotide, which reaction results in the production of a labeled polyphosphate; b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable species; and c) detecting the presence
30 of the detectable species.

Also provided is a method of detecting the presence of a nucleic acid sequence comprising the steps of: (a) conducting a nucleic acid polymerase reaction in the presence of at least one terminal-phosphate-labeled nucleotide having four or more

phosphate groups in the polyphosphate chain, which reaction results in the production of a labeled polyphosphate; and (b) detecting the labeled polyphosphate.

According to the above description the invention provides a method of sequencing a target region of a nucleic acid template, comprising:

- a) conducting a nucleic acid polymerization reaction on a solid support, by forming a reaction mixture, said reaction mixture including a nucleic acid template, a primer, a nucleic acid polymerizing enzyme, and one terminal-phosphate-labeled nucleoside polyphosphate with four or more phosphates, selected from a nucleoside with a natural base or a base analog and wherein a component of said reaction mixture or a complex of two or more of said components, is immobilized on said solid support, and said component or components are selected from the group consisting of said nucleic acid template, said primer, and said nucleic acid polymerizing enzyme, and said reaction results in production of labeled polyphosphate if said terminal-phosphate-labeled nucleoside polyphosphate contains a base complementary to the template base at the site of polymerization;
- b) detecting said labeled polyphosphate;
- c) continuing said polymerization reaction by adding a different terminal-phosphate-labeled nucleoside polyphosphate selected from the remaining natural bases or base analogs to said reaction mixture and repeating step b; and
- d) identifying said target region sequence from the identity and order of addition of terminal-phosphate labeled nucleoside polyphosphates resulting in production of said labeled polyphosphates.

In addition, the invention relates to a method of detecting the presence of a nucleic acid sequence comprising the steps of: (a) conducting a nucleic acid polymerase reaction in the presence of at least one terminal-phosphate-labeled nucleotide having four or more phosphate groups in the polyphosphate chain, which reaction results in the production of a labeled polyphosphate; (b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable species; and (c) detecting the presence of the detectable species.

A further aspect of the present invention relates to a method of quantifying a nucleic acid including the steps of: (a) conducting a nucleic acid polymerase reaction, wherein the reaction includes the reaction of a terminal-phosphate-labeled nucleotide, which reaction results in production of labeled polyphosphate; (b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable by-product species in an amount substantially proportional to the amount of nucleic acid; (c) measuring the detectable species; and (d) comparing the measurements using known standards to determine the quantity of nucleic acid.

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The invention further relates to a method of quantifying a DNA sequence including the steps of: (a) conducting a DNA polymerase reaction in the presence of a terminal-phosphate-labeled nucleotide, the reaction resulting in production of labeled polyphosphate; (b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable by-product species in amounts substantially proportional to the amount of the DNA sequence; (c) measuring the detectable species; and (d) comparing the measurements using known standards to determine the quantity of DNA.

20 Another aspect of the invention relates to a method for determining the identity of a single nucleotide in a nucleic acid sequence, which includes the steps of: (a) conducting a nucleic acid polymerase reaction in the presence of at least one terminal phosphate-labeled nucleotide, which reaction results in the production of labeled polyphosphate; (b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable species; (c) detecting the presence of the detectable species; and (d) identifying the nucleoside incorporated.

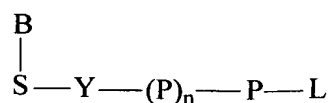
Also provided is a method for determining the identify of a single nucleotide in a nucleic acid sequence including the following steps: (a) conducting a nucleic acid polymeric reaction in the presence of at least one terminal-phosphate-labeled nucleotide having four or more phosphate groups in the polyphosphate chain, which reaction results in the production of labeled polyphosphate; (b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable species; (c)

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detecting the presence of said detectable species; and (d) identifying the nucleoside incorporated.

5 The present invention further includes a nucleic acid detection kit wherein the kit includes:

- a) at least one or more terminal-phosphate-labeled nucleotide according to formula:



wherein

10 P = phosphate (PO₃) and derivatives thereof;

n is 2 or greater;

Y is an oxygen or sulfur atom;

B is a nitrogen-containing heterocyclic base;

S is an acyclic moiety, carbocyclic moiety or sugar moiety;

15 P-L is a phosphorylated label which becomes independently detectable when the phosphate is removed,

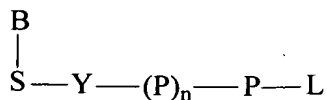
wherein L is an enzyme-activatable label containing a hydroxyl group, a sulfhydryl group or an amino group suitable for forming a phosphate ester, a thioester or a phosphoramidate linkage at the terminal

20 phosphate of a natural or modified nucleotide;

- b) at least one of DNA polymerase, RNA polymerase, or reverse transcriptase;
and
c) phosphatase.

25 The present invention also provides another nucleic acid detection kit comprising:

- a) at least one terminal-phosphate-labeled nucleoside polyphosphate according to the formula:



30 wherein

P=phosphate (PO₃) and derivatives thereof;

n is 3 or greater;

Y is an oxygen or sulfur atom;

B is a nitrogen-containing heterocyclic base;

5 S is an acyclic moiety, carbocyclic moiety or sugar moiety;

P-L is a phosphorylated label,

wherein L is a label containing a hydroxyl group, a haloalkyl group, a
sulfhydryl group or an amino group suitable for forming a phosphate
ester, a phosphonate, a thioester or a phosphoramidate linkage at the
10 terminal phosphate of a natural or modified nucleotide;

and

b) at least one enzyme is selected from the group consisting of DNA polymerase,
RNA polymerase and reverse transcriptase.

15 **Brief Description of the Drawings**

Figure 1 is a graph showing fluorescence obtained by polymerase utilization of a
gamma-phosphate-labeled ddGTP in a template-directed process in the presence of
phosphatase.

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Figure 2 is a graph showing fluorescence obtained by polymerase utilization of a
gamma-phosphate-labeled ddATP in a template-directed process in the presence of
phosphatase.

25 Figure 3 is a graph of relative fluorescence obtained upon sequential addition of a
terminal-phosphate-labeled nucleotide in the presence of phosphatase.

Figure 4 is a schematic of sequencing with terminal-phosphate labeled nucleoside
polyphosphates in a flow-through or stop-flow system.

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Detailed Description of the Preferred Embodiment

The term “nucleoside” as defined herein is a compound including a purine,
deazapurine, pyrimidine or modified base linked to a sugar or a sugar substitute, such

as a carbocyclic or acyclic moiety, at the 1' position or equivalent position and includes 2'-deoxy and 2'-hydroxyl, and 2', 3'-dideoxy forms as well as other substitutions.

5 The term “nucleotide” as used herein refers to a phosphate ester of a nucleoside, wherein the esterification site typically corresponds to the hydroxyl group attached to the C-5 position of the pentose sugar.

10 The term “oligonucleotide” includes linear oligomers of nucleotides or derivatives thereof, including deoxyribonucleosides, ribonucleosides, and the like. Throughout the specification, whenever an oligonucleotide is represented by a sequence of letters, the nucleotides are in the 5' → 3' order from left to right where A denotes deoxyadenosine, C denotes deoxycytidine, G denotes deoxyguanosine, and T denotes thymidine, unless noted otherwise.

15 The term “primer” refers to a linear oligonucleotide that anneals in a specific way to a unique nucleic acid sequence and allows for amplification of that unique sequence.

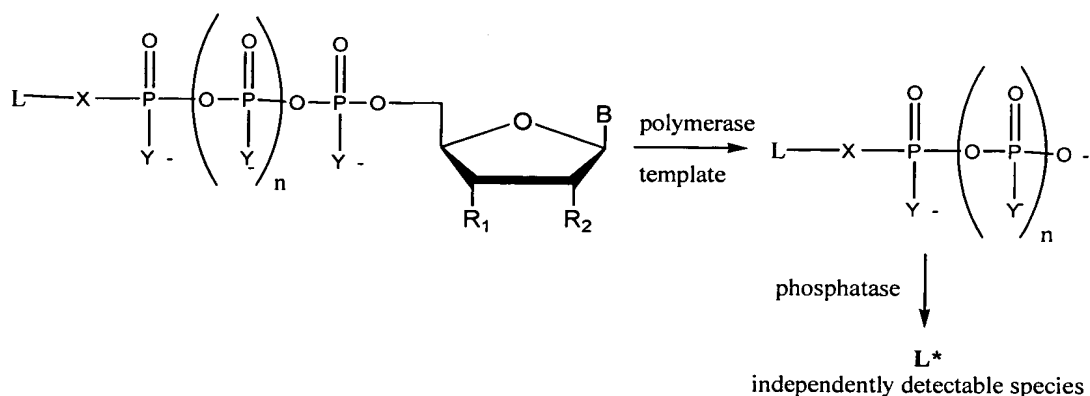
20 The phrase “target nucleic acid sequence” and the like refers to a nucleic acid whose sequence identity, or ordering or location of nucleosides is determined by one or more of the methods of the present invention.

25 The present invention relates to methods of sequencing a polynucleotide in a sample wherein a convenient assay is used for monitoring RNA or DNA synthesis via nucleic acid polymerase activity. RNA and DNA polymerases synthesize oligonucleotides via transfer of a nucleoside monophosphate from a nucleoside triphosphate (NTP) or deoxynucleoside triphosphate (dNTP) to the 3' hydroxyl of a growing oligonucleotide chain. The force which drives this reaction is the cleavage of an anhydride bond and the con-comitant formation of an inorganic pyrophosphate. 30 The present invention utilizes the finding that structural modification of the terminal-phosphate of the nucleotide does not abolish its ability to function in the polymerase reaction. The oligonucleotide synthesis reaction involves direct changes only at the α - and β - phosphoryl groups of the nucleotide, allowing nucleotides with modifications

at the terminal phosphate position to be valuable as substrates for nucleic acid polymerase reactions.

In certain embodiments, the polymerase is a DNA polymerase, such as DNA polymerase I, II, or III or DNA polymerase α , β , γ , or terminal deoxynucleotidyl transferase or telomerase. In other embodiments, suitable polymerases include, but are not limited to, a DNA dependent RNA polymerase, a primase, or an RNA dependant DNA polymerase (reverse transcriptase).

The methods provided by this invention utilize a nucleoside polyphosphate, such as a deoxynucleoside polyphosphate, dideoxynucleoside polyphosphate, carbocyclic nucleoside polyphosphate, or acyclic nucleoside polyphosphate analogue with an electrochemical label, mass tag, or a colorimetric dye, a chemiluminescent label, or a fluorescent label attached to the terminal-phosphate. When a nucleic acid polymerase uses this analogue as a substrate, an enzyme-activatable label would be present on the inorganic polyphosphate by-product of phosphoryl transfer. Cleavage of the polyphosphate product of phosphoryl transfer via phosphatase, leads to a detectable change in the label attached thereon. It is noted that while RNA and DNA polymerases are able to recognize nucleotides with modified terminal phosphoryl groups, the inventors have determined that this starting material is not a template for phosphatases. The scheme below shows the most relevant molecules in the methods of this invention; namely the terminal-phosphate-labeled nucleotide, the labeled polyphosphate by-product and the enzyme-activated label.



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In the scheme above, n is 1 or greater, R_1 and R_2 are independently H, OH, SH, SR, OR, F, Br, Cl, I, N_3 , NHR or NH_2 ; B is a nucleoside base or modified

heterocyclic base; X is O, S, or NH; Y is O, S, or BH₃; and L is a phosphatase activatable label which may be a chromogenic, fluorogenic, chemiluminescent molecule, mass tag or electrochemical tag. A mass tag is a small molecular weight moiety suitable for mass spectrometry that is readily distinguishable from other components due to a difference in mass. An electrochemical tag is an easily oxidizable or reducible species. It has been discovered that when n is 2 or greater, the nucleotides are significantly better substrates for polymerases than when n is 1. Therefore, in preferred embodiments, n is 2, 3 or 4, R1 and R2 are independently H or OH; X and Y are O; B is a nucleotide base and L is a label which may be a chromogenic, fluorogenic or a chemiluminescent molecule.

In one embodiment of the method of detecting the presence of a nucleic acid sequence provided herein, the steps include (a) conducting a nucleic acid polymerase reaction wherein the reaction includes a terminal-phosphate-labeled nucleotide wherein the polymerase reaction results in the production of labeled polyphosphate; (b) permitting the labeled polyphosphate to react with a phosphatase suitable to hydrolyze the phosphate ester and to produce a detectable species; and c) detecting the presence of a detectable species by suitable means. In this embodiment, the template used for the nucleic acid polymerase reaction may be a heteropolymeric or homopolymeric template. By terminal-phosphate-labeled nucleotide, it is meant throughout the specification that the labeled polyphosphate concomitantly released following incorporation of the nucleoside monophosphate into the growing nucleotide chain, may be reacted with the phosphatase to produce a detectable species. Other nucleotides included in the reaction which are substantially non-reactive to phosphatase may be, for example, blocked at the terminal-phosphate by a moiety which does not lead to the production of a detectable species. The nucleic acid for detection in this particular embodiment may include RNA, a natural or synthetic oligonucleotide, mitochondrial or chromosomal DNA.

The invention further provides a method of detecting the presence of a DNA sequence including the steps of (a) conducting a DNA polymerase reaction in the presence of a terminal-phosphate labeled nucleotide, which reaction results in the production of a labeled polyphosphate; (b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable species; and (c) detecting the

presence of said detectable species. The DNA sequence for detection may include DNA isolated from cells, chemically treated DNA such as bisulfite treated methylated DNA or DNA chemically or enzymatically synthesized according to methods known in the art. Such methods include PCR, and those described in DNA Structure Part A: Synthesis and Physical analysis of DNA, Lilley, D.M.J. and Dahlberg, J.E. (Eds.), Methods Enzymol., 211, Academic Press, Inc., New York (1992), which is herein incorporated by reference. The DNA sequence may further include chromosomal DNA and natural or synthetic oligonucleotides. The DNA may be either double- or single-stranded.

The methods of the invention may further include the step of including one or more additional detection reagents in the polymerase reaction. The one or more additional detection reagents are each independently capable of a response that is detectably different from each other and from the detectable species. For example, one or more of the one or more additional detection reagent may be an antibody.

Suitable nucleotides for addition as substrates in the polymerase reaction include nucleoside polyphosphates, such as including, but not limited to, deoxyribonucleoside polyphosphates, ribonucleoside polyphosphates, dideoxynucleoside polyphosphates, carbocyclic nucleoside polyphosphates and acyclic nucleoside polyphosphates and analogs thereof. Particularly desired are nucleotides containing 3, 4, 5 or 6 phosphate groups in the polyphosphate chain, where the terminal phosphate is labeled.

It is noted that in embodiments including terminal-phosphate-labeled nucleotides having four or more phosphates in the polyphosphate chain, it is within the contemplation of the present invention that the labeled polyphosphate by-product of phosphoryl transfer may be detected without the use of phosphatase treatment. For example, it is known that natural or modified nucleoside bases, particularly guanine, can cause quenching of fluorescent markers. Therefore, in a terminal-phosphate-labeled nucleotide, the label may be partially quenched by the base. Upon incorporation of the nucleoside monophosphate, the label polyphosphate by-product may be detected due to its enhanced fluorescence. Alternatively, it is possible to physically separate the labeled polyphosphate product by chromatographic separation

methods before identification by fluorescence, color, chemiluminescence, or electrochemical detection. In addition, mass spectrometry could be used to detect the products by mass difference.

5 The methods of the present invention may include conducting the polymerase reaction in the presence of at least one of DNA or RNA polymerase. Suitable nucleic acid polymerases may also include primases, telomerases, terminal deoxynucleotidyl transferases, and reverse transcriptases. A nucleic acid template may be required for the polymerase reaction to take place and may be added to the polymerase reaction
10 solution. It is anticipated that all of the steps (a), (b) and (c) in the detection methods of the present invention could be run concurrently using a single, homogenous reaction mixture, as well as run sequentially.

 It is well within the contemplation of the present invention that nucleic acid
15 polymerase reactions may include amplification methods that utilize polymerases. Examples of such methods include polymerase chain reaction (PCR), rolling circle amplification (RCA), and nucleic acid sequence based amplification (NASBA). For e.g., wherein the target molecule is a nucleic acid polymer such as DNA, it may be detected by PCR incorporation of a gamma-phosphate labeled nucleotide base such as
20 adenine, thymine, cytosine, guanine or other nitrogen heterocyclic bases into the DNA molecule. The polymerase chain reaction (PCR) method is described by Saiki et al in Science Vol. 239, page 487, 1988, Mullis et al in U.S. Patent 4,683,195 and by Sambrook, J. et al. (Eds.), Molecular Cloning, second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1980), Ausubel, F.M. et al. (Eds.),
25 Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY (1999), and Wu, R. (Ed.), Recombinant DNA Methodology II, Methods in Enzymology, Academic Press, Inc., NY, (1995). Using PCR, the target nucleic acid for detection such as DNA is amplified by placing it directly into a reaction vessel containing the PCR reagents and appropriate primers. Typically, a primer is selected which is
30 complimentary in sequence to at least a portion of the target nucleic acid.

 It is noted that nucleic acid polymerase reactions suitable for conducting step (a) of the methods of the present invention may further include various RCA methods of amplifying nucleic acid sequences. For example, those disclosed in U.S. Patent

5,854,033 to Lizardi, Paul M., incorporated herein by reference, are useful.

Polymerase reactions may further include the nucleic acid sequence based amplification (NASBA) wherein the system involves amplification of RNA, not DNA, and the amplification is iso-thermal, taking place at one temperature (41°C).

5 Amplification of target RNA by NASBA involves the coordinated activities of three enzymes: reverse transcriptase, Rnase H, and T7 RNA polymerase along with oligonucleotide primers directed toward the sample target RNA. These enzymes catalyze the exponential amplification of a target single-stranded RNA in four steps: extension, degradation, DNA synthesis and cyclic RNA amplification.

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Methods of RT-PCR, RCA, and NASBA generally require that the original amount of target nucleic acid is indirectly measured by quantification of the amplification products. Amplification products are typically first separated from starting materials via electrophoresis on an agarose gel to confirm a successful
15 amplification and are then quantified using any of the conventional detection systems for a nucleic acid such as detection of fluorescent labels, enzyme-linked detection systems, antibody-mediated label detection and detection of radioactive labels. In contrast, the present method eliminates the need to separate products of the polymerase reaction from starting materials before being able to detect these products.

20 For example, in the present invention, a reporter molecule (fluorescent, chemiluminescent or a chromophore) or other useful molecule is attached to the nucleotide in such a way that it is undetectable under certain conditions when masked by the phosphate attachment. However, following the incorporation of the nucleotide into the growing oligonucleotide chain and phosphatase treatment of the reaction, the
25 label is detectable under those conditions. For example, if the hydroxyl group on the side of the triple ring structure of 1,3-dichloro-9,9-dimethyl-acridine-2-one (DDAO) is attached to the terminal-phosphate position of the nucleotide, the DDAO does not fluoresce at 659 nm. Once the nucleoside monophosphate is incorporated into DNA, the other product, DDAO polyphosphate (which also does not fluoresce at 659 nm) is
30 a substrate for phosphatase. Once de-phosphorylated to form DDAO, the dye moiety will become fluorescent at 659 nm and hence detectable. The specific analysis of the polyphosphate product can be carried out in the polymerase reaction solution, eliminating the need to separate reaction products from starting materials. This scheme allows for the detection and, optionally, quantification of nucleic acids

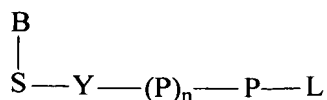
formed during polymerase reactions using routine instrumentation such as spectrophotometers.

In the methods described above, the polymerase reaction step may further include conducting the polymerase reaction in the presence of a phosphatase, which converts labeled polyphosphate by-product to the detectable label. As such, a convenient assay is established for detecting the presence of a nucleic acid sequence that allows for continuous monitoring of detectable species formation. This represents a homogeneous assay format in that it can be performed in a single tube.

One format of the assay methods described above may include, but is not limited to, conducting the polymerase reaction in the presence of a single type of terminal-phosphate-labeled nucleotide capable of producing a detectable species, for example terminal-phosphate-modified ATP, wherein all other nucleotides are substantially non-reactive to phosphatase, but yield non-detectable species.

In another assay format, the polymerase reaction may be conducted in the presence of more than one type of terminal-phosphate-labeled nucleotide, each type capable of producing a uniquely detectable species. For example, the assay may include a first nucleotide (e.g., adenosine polyphosphate) that is associated with a first label which when liberated enzymatically from the inorganic polyphosphate by-product of phosphoryl transfer, emits light at a first wavelength and a second nucleotide (e.g., guanosine polyphosphate) associated with a second label that emits light at a second wavelength. Desirably, the first and second wavelength emissions have substantially little or no overlap. It is within the contemplation of the present invention that multiple simultaneous assays based on nucleotide sequence information can thereafter be derived based on the particular label released from the polyphosphate.

In one aspect of the methods of detecting the presence of a nucleic acid sequence described above, the terminal-phosphate-labeled nucleotide may be represented by the formula:



wherein

P = phosphate (PO₃) and derivatives thereof;

5 n is 2 or greater;

Y is an oxygen or sulfur atom;

B is a nitrogen-containing heterocyclic base;

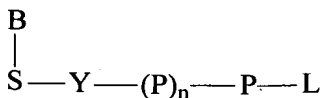
S is an acyclic moiety, carbocyclic moiety or sugar moiety;

10 P-L is a phosphorylated label which becomes independently detectable when the phosphate is removed,

wherein L is an enzyme-activatable label containing a hydroxyl group, a sulfhydryl group or an amino group suitable for forming a phosphate ester, a thioester or a phosphoramidate linkage at the terminal phosphate of a natural or modified nucleotide.

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In another aspect of the methods of detecting the presence of a nucleic acid sequence described above wherein the detectable species is the labeled polyphosphate, the terminal-phosphate-labeled nucleotide may be represented by the formula:



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wherein

P = phosphate (PO₃) and derivatives thereof;

n is 3 or greater;

Y is an oxygen or sulfur atom;

25 B is a nitrogen-containing heterocyclic base;

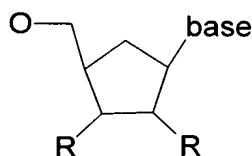
S is an acyclic moiety, carbocyclic moiety or sugar moiety;

P-L is a phosphorylated label,

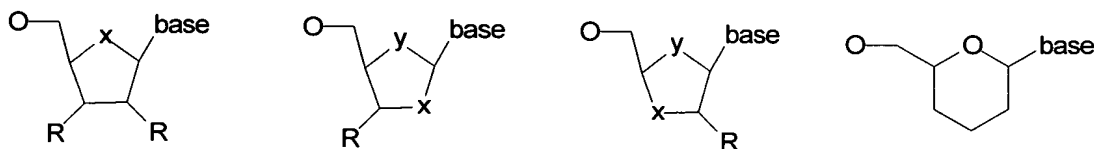
wherein L is a label containing a hydroxyl group, a haloalkyl group, a sulfhydryl group or an amino group suitable for forming a phosphate

ester, a phosphonate, a thioester or a phosphoramidate linkage at the terminal phosphate of a natural or modified nucleotide.

For purposes of the methods of the present invention, useful carbocyclic
5 moieties have been described by Ferraro, M. and Gotor, V. in Chem Rev. 2000, volume 100, 4319-48. Suitable sugar moieties are described by Joeng, L.S. et al., in J Med. Chem. 1993, vol. 356, 2627-38; by Kim H.O. et al., in J Med. Chem. 1993, vol. 36, 30-7; and by Eschenmosser A., in Science 1999, vol. 284, 2118-2124. Moreover, useful acyclic moieties have been described by Martinez, C.I., et al., in Nucleic Acids
10 Research 1999, vol. 27, 1271-1274; by Martinez, C.I., et al., in Bioorganic & Medicinal Chemistry Letters 1997, vol. 7, 3013-3016; and in U.S. Patent 5,558,91 to Trainer, G.L. Structures for these moieties are shown below, where for all moieties R may be H, OH, NHR, F, N₃, SH, SR, OR lower alkyl and aryl; for the sugar moieties X and Y are independently O, S, or NH; and for the acyclic moieties, X = O, S, NH,
15 NR.

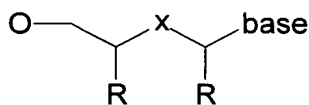


Carbocyclic Moieties



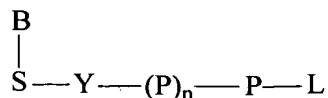
Sugar Moieties

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Acyclic Moieties

In certain embodiments, the sugar moiety in the formula



may be selected from the following: ribosyl, 2'-deoxyribosyl, 3'-deoxyribosyl, 2', 3'-didehydrodideoxyribosyl, 2',3'-dideoxyribosyl, 2'- or 3'-alkoxyribosyl, 2'- or 3'-aminoribosyl, 2'- or 3'-fluororibosyl, 2'- or 3'-mercaptoribosyl, 2'- or 3'-alkylthioribosyl, acyclic, carbocyclic and other modified sugars.

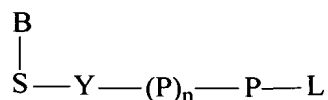
Moreover, in above formula, the base may include uracil, thymine, cytosine, 5-methylcytosine, guanine, 7-deazaguanine, hypoxanthine, 7-deazahypoxanthine, adenine, 7-deazaadenine, 2, 6-diaminopurine or analogs thereof.

For the preferred embodiments of the current invention, where the the label is activated after phosphatase treatment, the label attached at the terminal-phosphate position in the terminal-phosphate-labeled nucleotide may be selected from the group consisting of 1,2-dioxetane chemiluminescent compounds, fluorogenic dyes, chromogenic dyes, mass tags and electrochemical tags. This would allow the detectable species to be detectable by the presence of any one of color, fluorescence emission, chemiluminescence, mass change, electrochemical detection or a combination thereof. Some of the dyes useful in the present invention are shown in table 1. It is within the scope of current invention to use other dyes that are derivatives of these as well as others that undergo a detectable change in a physical or chemical property after removal of the phosphate group.

Table 1: Examples of detectable label moieties that become independently detectable after removal of phosphate residues	
9H-(1,3-dichloro-9,9-dimethyl-7-hydroxyacridin-2-one)	
9H-(9,9-dimethyl-7-hydroxyacridin-2-one)	
9H-(1,3-dibromo-9,9-dimethyl-7-hydroxyacridin-2-one)	
Resorufin	
Umbelliferone (7-hydroxycoumarin)	
4-Methylumbelliferone	
4-Trifluoromethylumbelliferone	
3-Cyanoumbelliferone	

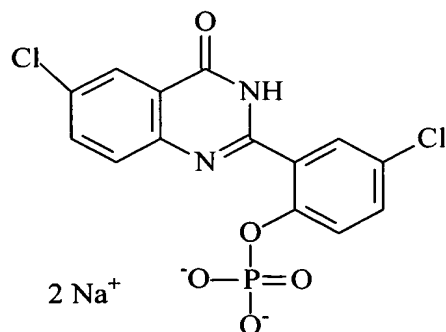
3-Phenylumbelliferone
3,4-Dimethylumbelliferone
3-Acetylumbelliferone
6-Methoxyumbelliferone
SNAFL™
Fluorescein ethyl ether
Naphthofluorescein
Naphthofluorescein ethyl ether
SNARF™
Rhodol green™
Meso-Hydroxymonocarbocyanine
Meso-hydroxytricarbocyanine
Meso-hydroxydicarbocyanine
Bis-(1,3-dibutylbarbituric acid)pentamethine oxonol
1-Ethyl-2-(naphthyl-1-vinylene)-3,3-dimethyl-indolinium salt
A Merrocyanine
2-Hydroxy-5'-chloro-phenyl4-(3H)-6- chloro-quinazolinone
Trifluoroacetyl-rhodol
Acetyl-rhodol
8-Hydroxy-2H-dibenz(b,f)azepin-2-one
8-hydroxy-11,11-dimethyl-11H-dibenz(b,e)(1,4)oxazepin-2-one
2-hydroxy-11,11-dimethyl-11H-
Hydroxypyrene
Dibenz(b,e)(1,4)oxazepin-8-one

Wherein the phosphorylated label in the formula



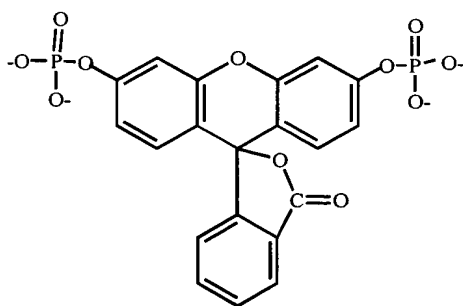
- is a fluorogenic moiety, it is desirably selected from one of the following (all shown as the phosphomonoester):
- 5 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone, sold under the trade name ELF 97 (Molecular Probes, Inc.),
 - fluorescein diphosphate (tetraammonium salt), fluorescein 3'(6')-O-alkyl-6'(3')-phosphate, 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)phosphate (diammonium salt), 4-methylumbelliferyl phosphate (free acid), resorufin phosphate, 4-
 - 10 trifluoromethylumbelliferyl phosphate, umbelliferyl phosphate, 3-cyanoubelliferyl phosphate, 9,9-dimethylacridin-2-one-7-yl phosphate, 6,8-difluoro-4-

methylumbelliferyl phosphate and derivatives thereof. Structures of these dyes are shown below:

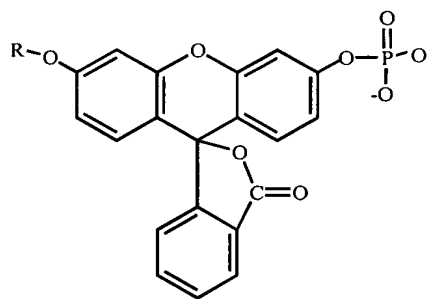


2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone

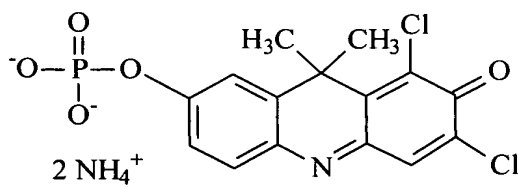
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fluorescein diphosphate

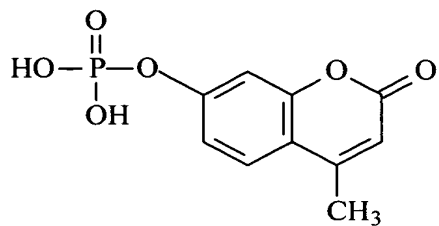


fluorescein 3'-(6')-O-alkyl-6'-(3')-phosphate

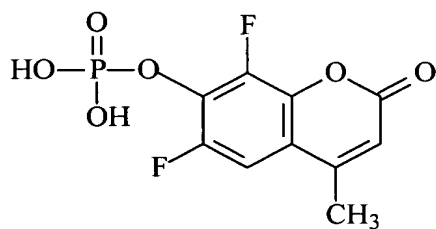


10

9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)phosphate(diammonium salt)

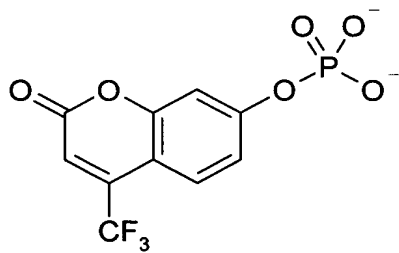


4-methylumbelliferyl phosphate



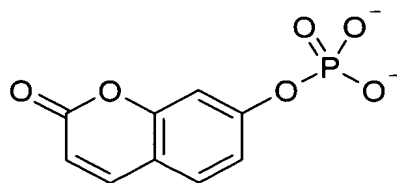
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6, 8-difluoro-4-methylumbelliferyl phosphate

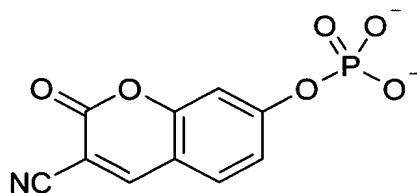


4-Trifluoromethylumbelliferyl phosphate

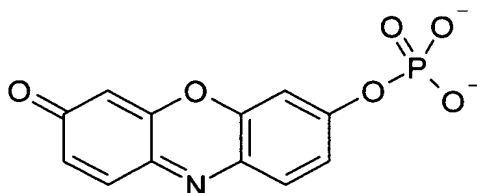
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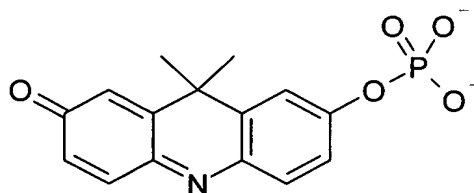
Umbelliferyl phosphate



3-Cyanoumbelliferyl phosphate

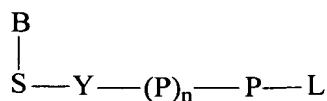


Resorufin phosphate

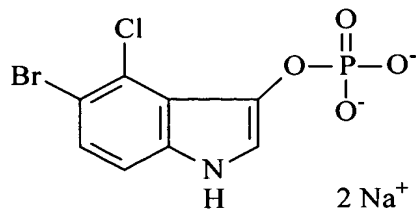


9, 9-dimethylacridin-2-one-7-yl phosphate

Wherein the phosphorylated label moiety in the formula

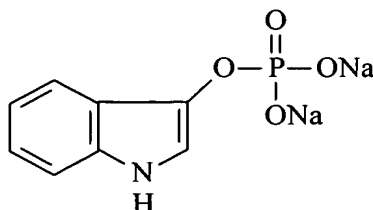


is a chromogenic moiety, it may be selected from the following: 5-bromo-4-chloro-3-indolyl phosphate, 3-indoxyl phosphate, p-nitrophenyl phosphate and derivatives thereof. The structures of these chromogenic dyes are shown as the phosphomonoesters below:

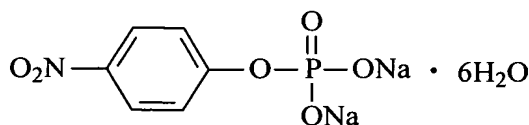


5-bromo-4-chloro-3-indolyl phosphate (disodium salt)

5



3-indolyl phosphate (disodium salt)



p-nitrophenyl phosphate

10

The moiety at the terminal-phosphate position may further be a chemiluminescent compound wherein it is desired that it is a phosphatase-activated 1,2-dioxetane compound. The 1,2-dioxetane compound may include, but is not limited to, disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'-(5-chloro-
 15)tricyclo[3,3,1-13,7]-decan]-1-yl)-1-phenyl phosphate, sold under the trade name CDP-Star (Tropix, Inc., Bedford, MA), chloroadamant-2'-ylidenemethoxyphenoxy phosphorylated dioxetane, sold under the trade name CSPD (Tropix), and 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)phenyl-1,2-dioxetane, sold under the trade name AMPPD (Tropix). The structures of these commercially available
 20 dioxetane compounds are disclosed in US patents 5,582,980, 5,112,960 and 4,978,614, respectively, and are incorporated herein by reference.

For embodiments of the current invention that involve detection of labeled polyphosphates, any fluorescent dye or colored dye from known classes of fluorescent and colored dyes, e.g. xanthenes, cyanines, porphyrins, coumarines, bodipy dyes, merrocyanines, pyrenes, azo dyes, etc., that are appropriately functionalized to attach to a phosphate can be used. These dyes are well known and are available from a number of commercial sources. A few examples of dyes that are readily detectable as labeled polyphosphates are shown in Table 2.

Table 2: Examples of detectable species that are detectable as labeled polyphosphates

	Rhodamine green carboxylic acid	Carboxy-fluorescein
	Pyrene	Dansyl
	Bodipy	Dimethylamino-coumarin carboxylic acid
15	Eosin-5-isothiocyanate	Methoxycoumarin carboxylic acid
	Texas Red	Oregon Green™ 488 carboxylic acid
	ROX	TAMRA
	Anthracene-isothiocyanate	Cy3
20	Cy3.5	Cy5
	Cy5.5	Anilinonaphthalene- sulfonic acid

The methods described above may further include the step of quantifying the nucleic acid sequence. In a related aspect, the detectable species may be produced in amounts substantially proportional to the amount of an amplified nucleic acid sequence. The step of quantifying the nucleic acid sequence is desired to be done by comparison of spectra produced by the detectable species with known spectra.

The present invention further provides a method of sequencing a nucleic acid sequence by a) immobilizing one of the key components of the sequencing reaction, such as polymerizing enzyme, primer, template or a complex formed by mixing 2 or more of these components, b) allowing the hybridization to proceed unless it was already done prior to step a, c) incubating in the presence of a nucleic acid polymerizing enzyme, a phosphatase and a terminal-phosphate-labeled nucleoside polyphosphate, which reaction produces labeled polyphosphate if the nucleotide present is complementary to the target sequence at the site of polymerization. The labeled polyphosphate then reacts with phosphatase or a phosphate or polyphosphate

transferring enzyme to produce free label with a signal readily distinguishable from the phosphate bound dye. If the nucleotide added is not complementary to the target sequence at the site of polymerization, no polymerization takes place and no free label is produced. Thus, formation of free label identifies the base added and hence the target sequence. After sufficient time is allowed for the polymerization reaction, which may range from milliseconds to several minutes, and detecting the presence or absence of signal, solid support may be separated from solution by any of the means known in the art, including but not limited to washing, filtration, centrifugation, decantation, etc., and next nucleotides may be added in the presence of fresh polymerase (if needed) and phosphatase. The order of addition of terminal-phosphate labeled nucleotides that actually result in the formation of a detectable species determines the sequence of the target nucleic acid. It should be complementary to the bases added. It should also be noted that phosphatase may be added after the polymerization has already proceeded.

In one aspect of the invention, a target nucleic acid may be probed for the presence of a known sequence according to the method described above. In this case, one may choose to add terminal-phosphate labeled nucleoside polyphosphate in the exact order that is supposed to result in the incorporation of complementary bases. In other words, if the target sequence is expected to be ACGGTA, the terminal-labeled nucleoside polyphosphates may be added in the order TGCCAT.

In another aspect one may choose to add terminal-phosphate labeled nucleoside polyphosphates in a preset order and repeat this order in a cyclic manner. This may be done whether the target nucleic acid is being probed for a known sequence or unknown sequence. For example, one could add terminal-phosphate labeled nucleoside polyphosphates in the order AGCT and repeat this order any number of cycles.

As discussed later in more detail, these terminal-phosphate labeled nucleoside polyphosphates may contain the natural bases or analogs thereof as long as the complementarity is preserved.

The present invention further provides methods of sequencing a target sequence using the steps described above in a continuous flow or a stop-flow system, where the immobilized material is held in place by any one of the means known in the art and different reagents and buffers are pumped in to the system at one end and exit the system at the other end. Reagents and buffers may flow continuously or may be held in place for certain time to allow for the polymerization reaction and phosphatase hydrolysis to proceed. An illustration of the process is presented in Figure 4.

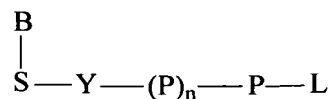
In one embodiment, the invention provides a method of quantifying a nucleic acid including the steps of: (a) conducting a nucleic acid polymerase reaction, the polymerase reaction including the reaction of a terminal-phosphate-labeled nucleotide, wherein the reaction results in the production of labeled polyphosphate; (b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable by-product species in an amount substantially proportional to the amount of the nucleic acid to be quantified; (c) measuring the detectable species; and (d) comparing the measurements using known standards to determine the quantity of the nucleic acid. In this embodiment of the method of quantifying a nucleic acid, the nucleic acid to be quantified may be RNA. The nucleic acid may further be a natural or synthetic oligonucleotide, chromosomal DNA, or DNA.

The invention further provides a method of quantifying a DNA sequence including the steps of: (a) conducting a DNA polymerase reaction in the presence of a terminal-phosphate-labeled nucleotide wherein the reaction results in the production of labeled polyphosphate; (b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable by-product species in amounts substantially proportional to the amount of the DNA sequence to be quantified; (c) measuring the detectable species; and (d) comparing measurements using known standards to determine the quantity of DNA. In this embodiment, the DNA sequence for quantification may include natural or synthetic oligonucleotides, or DNA isolated from cells including chromosomal DNA.

In each of these methods of quantifying a nucleic acid sequence described above, the polymerase reaction step may further include conducting the polymerase reaction in the presence of a phosphatase. As described earlier in the specification,

this would permit real-time monitoring of nucleic acid polymerase activity and hence, real-time detection of a target nucleic acid sequence for quantification.

The terminal-phosphate-labeled nucleotide useful for the methods of
5 quantifying the nucleic acid sequence provided herein may be represented by the formula:



The most preferred terminal-phosphate labeled nucleoside polyphosphates of
10 the formula for the method of quantifying the nucleic acid sequence provided herein are those with enzyme-activatable label. The enzyme-activatable label becomes detectable through the enzymatic activity of phosphatase which changes the phosphate ester linkage between the label and the terminal-phosphate of a natural or modified nucleotide in such a way to produce a detectable species. The detectable
15 species is detectable by the presence of any one of or a combination of color, fluorescence emission, chemiluminescence, mass difference or electrochemical potential. As already described above, the enzyme-activatable label may be a 1,2-dioxetane chemiluminescent compound, fluorescent dye, chromogenic dye, a mass tag or an electrochemical tag or a combination thereof. Suitable labels are the same as
20 those described above.

As will be described in further detail in the Example Section, the present invention provides methods for determining the identity of a single nucleotide in a target nucleic acid sequence. These methods include the steps of: (a) conducting a
25 nucleic acid polymerase reaction in the presence of at least one terminal phosphate-labeled nucleotide, which reaction results in the production of labeled polyphosphate; (b) permitting the labeled polyphosphate to react with a phosphatase to produce a detectable species; (c) detecting the presence of the detectable species; and (d) identifying the nucleoside incorporated. In desired embodiments, the terminal
30 phosphate-labeled nucleotide includes four or more phosphates in the polyphosphate chain.

Another aspect of the invention relates to a nucleic acid detection kit including:

- a) at least one or more terminal-phosphate-labeled nucleotides according to formula:



wherein

P=phosphate (PO₃) and derivatives thereof;

n is 2 or greater;

Y is an oxygen or sulfur atom;

10 B is a nitrogen-containing heterocyclic base;

S is an acyclic moiety, carbocyclic moiety or sugar moiety;

P-L is a phosphorylated label which preferably becomes independently detectable when the phosphate is removed,

15 wherein L is an enzyme-activatable label containing a hydroxyl group, a sulfhydryl group or an amino group suitable for forming a phosphate ester, a thioester or a phosphoramidate linkage at the terminal phosphate of a natural or modified nucleotide;

- b) at least one of DNA polymerase, RNA polymerase or reverse transcriptase; and

20 c) phosphatase.

Yet another aspect of the invention relates to a nucleic acid detection kit comprising:

- a) at least one terminal-phosphate-labeled nucleoside polyphosphate according to the formula:



wherein

P=phosphate (PO₃) and derivatives thereof;

n is 3 or greater;

30 Y is an oxygen or sulfur atom;

B is a nitrogen-containing heterocyclic base;

S is an acyclic moiety, carbocyclic moiety or sugar moiety;

P-L is a phosphorylated label,

wherein L is a label containing a hydroxyl group, a haloalkyl group, a
sulfhydryl group or an amino group suitable for forming a phosphate
ester, a phosphonate, a thioester or a phosphoramidate linkage at the
terminal phosphate of a natural or modified nucleotide;

and

- b) at least one enzyme is selected from the group consisting of DNA polymerase,
RNA polymerase and reverse transcriptase.

The sugar moiety in the terminal-phosphate-labeled nucleotide included in
these kits may include, but is not limited to ribosyl, 2'-deoxyribosyl, 3'-deoxyribosyl,
2', 3'-dideoxyribosyl, 2', 3'-didehydrodideoxyribosyl, 2'- or 3'-alkoxyribosyl, 2'- or
3'-aminoribosyl, 2'- or 3'-fluororibosyl, 2'- or 3'-mercaptoribosyl, 2'- or 3'-
alkylthioribosyl, acyclic, carbocyclic and other modified sugars.

The base may be, but is not limited to uracil, thymine, cytosine, 5-
methylcytosine, guanine, 7-deazaguanine, hypoxanthine, 7-deazahypoxanthine,
adenine, 7-deazaadenine and 2,6-diaminopurine and analogs thereof.

Furthermore, as described above, the enzyme-activatable label may be a 1,2-
dioxetane chemiluminescent compound, fluorescent dye, chromogenic dye, a mass
tag, an electrochemical tag or a combination thereof. Suitable compounds for
conjugation at the terminal-phosphate position of the nucleotide are the same as those
described above.

EXAMPLES

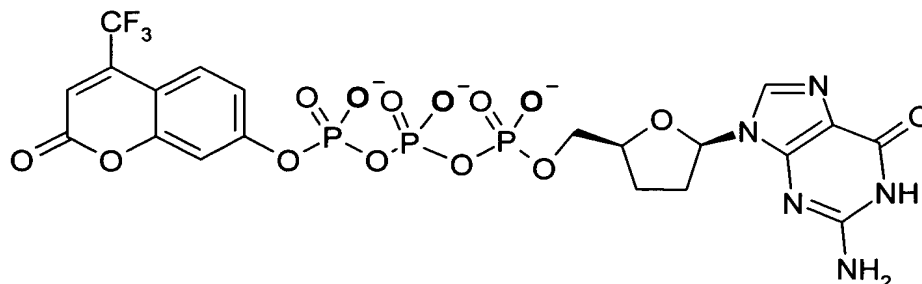
The following examples are for illustration purposes only and should not be
used in any way to limit the appended claims.

Example 1

Preparation of γ -(4-trifluoromethylcoumarinyl)ddGTP (γ CF₃Coumarin-ddGTP)

ddGTP (200 μ l of 46.4 mM solution, purity >96%) was coevaporated with
5 anhydrous dimethylformamide (DMF, 2x 0.5 ml). To this dicyclohexylcarbodiimide
(DCC, 9.6 mg, 5 eq.) was added and mixture was again coevaporated with anhyd.
DMF (0.5 ml). Residue was taken in anhyd. DMF (0.5 ml) and mixture was allowed
to stir overnight. There was still ca 20% uncyclized triphosphate (could be from
hydrolysis of cyclic trimetaphosphate on the column). To the mixture another 2 eq. of
10 DCC was added and after stirring for 2h, 7-hydroxy-4-trifluoromethyl coumarin (4-
trifluoromethylumbelliferone, 42.7 mg, 20 eq.) and triethylamine (26 μ l, 20 eq.) were
added and mixture was stirred at RT. After 2 days, HPLC (0-30% acetonitrile in
0.1M triethylammonium acetate (TEAA) in 15 minutes, 30-50 % acetonitrile in 5 min
and 50-100% acetonitrile in 10 minutes, C18 3.9x150 mm column, flow rate 1
15 ml/minute) showed a new product at 9.7 min and starting cyclic triphosphate (ratio of
77 to 5 at 254 nm). Mixture was allowed to stir for another day. P-31 NMR showed
gamma labeled nucleoside-triphosphate as the main component of reaction mixture.
Reaction mixture was concentrated on rotary evaporator. Residue was extracted with
water (5x1 ml). HPLC showed a purity of 82% at 254 nm and 81% at 335 nm.
20 Combined aq solution was conc. on rotary evaporator and redissolved in water (1 ml).
It was purified on 1 inch x 300 cm C18 column using 0-30% acetonitrile in 0.1M
triethylammonium bicarbonate (TEAB, pH 8.3) in 30 min and 30-50% acetonitrile in
10 min, 15 ml/min flow rate. Product peak was collected in 3 fractions. Fraction 1
was repurified using the same preparative HPLC method as above except the pH of
25 the TEAB buffer was reduced to 6.7 by bubbling CO₂. Product peak was
concentrated and coevaporated with MeOH (2 times) and water (1 time). Sample was
dissolved in 1 ml water. HPLC showed a purity of > 99% at 254 and 335 nm. UV
showed a conc. of 2.2 mM assuming an extinction coeff. of 11,000 at 322 nm (
reported for beta galactoside derivative of 7-hydroxy-4-trifluoromethylcoumarin,
30 Molecular Probes Catalog). MS: M- = 702.18 (calc 702.31), UV λ A = 253, 276 &
322 nm. The trifluorocoumarin dye attached to the gamma phosphate of ddGTP is
fluorescent with an excitation maximum of 322 nm and an emission maximum of
about 415 nm. Upon hydrolysis of the phosphate ester to release the free coumarin

dye, the spectrum changes with excitation maximum of about 385 nm and emission maximum of about 502 nm. This change is readily detected by simple fluorescence measurements or color change. Synthesis of gamma nucleotides has been generally described by Arzumanov, A. et al. in J Biol Chem (1996) Oct 4; 271 (40): 24389-94.



γ -(4-trifluoromethylcoumarinyl)dideoxyguanosine-5'-triphosphate
(γ CF₃Coumarin-ddGTP)

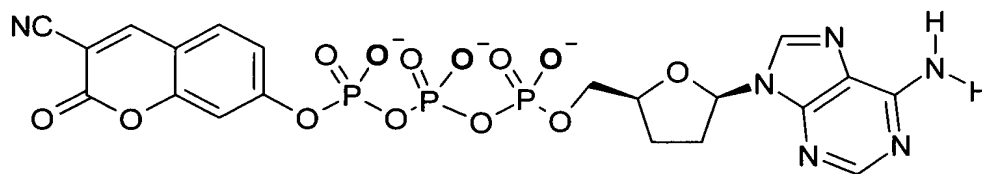
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Example 2

Preparation of γ - (3-Cyanocoumarinyl)ddATP (γ CNCoumarin-ddATP)

- 10 ddATP (100 μ l of 89 mM solution, >96%) was coevaporated with anhydrous DMF (2x 1 ml). To this DCC (9.2 mg, 5 eq.) was added and mixture was again coevaporated with anhydrous DMF (1 ml). Residue was taken in anhydrous DMF (0.5 ml) and reaction was stirred at rt. After overnight 7-hydroxy-3-cyanocoumarin (33.3 mg, 20 eq.) and TEA (25 μ l, 20 eq.), were added and mixture was stirred at RT .
- 15 After 1 day, a major product (55% at 254 nm) was observed 8.1 min with another minor product at 10 min (~10%). No significant change occurred after another day. Reaction mixture was concentrated on rotary evaporator and residue was extracted with 3x2 ml water and filtered. Aq solution was concentrated and purified on C-18 using 0-30% acetonitrile in 0.1M TEAB (pH 6.7) in 30 min and 30-50% acetonitrile
- 20 in 10 min, flow rate 15 ml/min. Main peak was collected in 3 fractions. HPLC of the main peak (fr. 2) showed a purity of 95.6% at 254 nm and 98.1% at 335 nm. It was concentrated on rotary evaporator (at RT), coevaporated with MeOH (2x) and water (1x). Residue was dissolved in 0.5 ml water. A 5 μ l sample was diluted to 1 ml for UV analysis. A_{346 nm} = 0.784. Assuming an extinction coeff. of 20,000 (reported

for 7-ethoxy-3-cyanocoumarin, Molecular Probes Catalog), concentration = 7.84 mM. Yield = 3.92 μ mol, 44%. Sample was repurified on C-18 column using same method as above. Sample peak was collected in 3 fractions. Fractions 2 & 3, with >98% purity at 254 nm and >99.5% purity at 340 nm, were combined. After concentration, residue was coevaporated with MeOH (2x) and water (1x). Sample was dissolved in water (1 ml) to give a 2.77 mM solution. MS: M- = 642.98 au (calc 643.00 au), UV λ A = 263 & 346 nm The cyanocoumarin dye attached to the gamma phosphate of ddATP is fluorescent with an excitation maximum of 346 nm and an emission maximum of about 411 nm. Upon hydrolysis of the phosphate ester to release the free coumarin dye, the spectrum changes with excitation maximum of about 408 nm and emission maximum of about 450 nm. This change is readily detected by simple fluorescence measurements or color change. Synthesis of gamma nucleotides has been generally described by Arzumanov, A, et al in J Biol Chem. (1996) Oct 4;271(40):24389-94.



γ -(3-cyanocoumarinyl)dideoxyadenosine-5'-triphosphate
(γ CNCoumarin-ddATP)

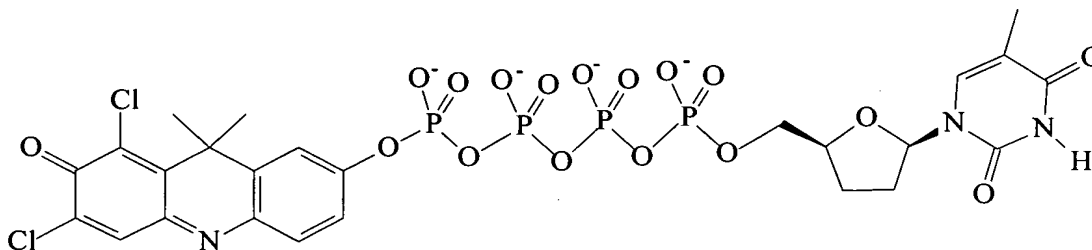
Example 3

Preparation of δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)-dideoxythymidine-5'-tetraphosphate (ddT4P-DDAO)

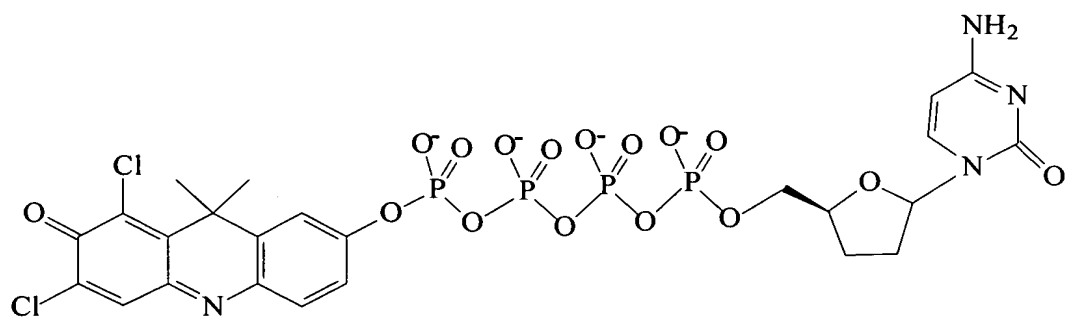
ddTTP (100 μ l of 80 mM solution) was coevaporated with anhydrous dimethylformamide (DMF, 2x 1 ml). To this dicyclohexylcarbodiimide (8.3 mg, 5 eq.) was added and the mixture was again coevaporated with anhydrous DMF (1 ml). Residue was taken in anhydrous DMF (1 ml) and reaction was stirred at room temperature overnight. HPLC showed mostly cyclized triphosphate (~82%). Reaction mixture was concentrated and residue was washed with anhydrous diethyl ether 3x. It was redissolved in anhydrous DMF and concentrated to dryness on

rotavap. Residue was taken with DDAO-monophosphate, ammonium salt (5 mg, 1.5 eq.) in 200 μ l anhydrous DMF and stirred at 40°C over the weekend. HPLC showed formation of a new product with desired UV characteristics at 11.96 min. (HPLC Method: 0.30% acetonitrile in 0.1M triethylammonium acetate (pH 7) in 15 min, and 30-50% acetonitrile in 5 min, Novapak C-18 3.9x150 mm column, 1 ml/min). LCMS (ES-) also showed a major mass peak 834 for M-1 peak. Reaction mixture was concentrated and purified on Deltapak C18, 19x 300 mm column using 0.1M TEAB (pH 6.7) and acetonitrile. Fraction with product was repurified by HPLC using the same method as described above. Fraction with pure product was concentrated, coevaporated with MeOH (2x) and water (1x). Residue was dissolved in water (1.2 ml) to give a 1.23 mM solution. HPCL purity at 254 nm > 97.5%, at 455 nm > 96%; UV λ A = 267 nm and 455 nm; MS: M-1 = 834.04 (calc 833.95).

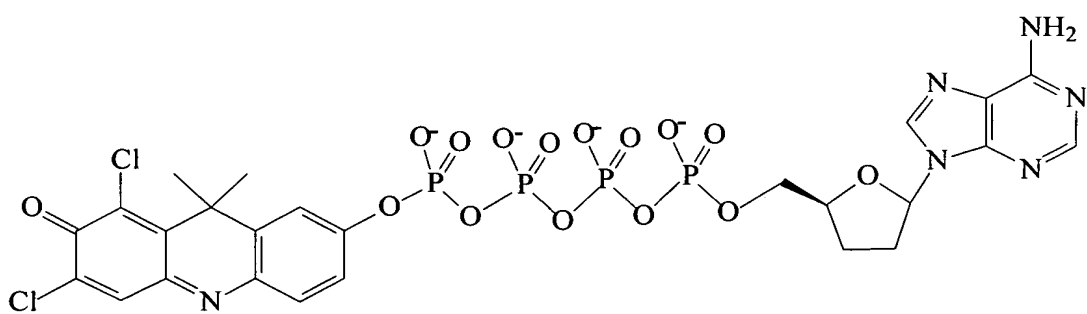
δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)-dideoxycytidine-5'-tetraphosphate (ddC4P-DDAO), δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-dideoxyadenosine-5'-tetraphosphate (ddA4P-DDAO) and δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-y-YL)-dideoxyguanosine-5'-tetraphosphate (ddG4P-DDAO) were synthesized and purified in a similar fashion. Analysis of these purified compounds provided the following data: ddC4P-DDAO: UV λ A = 268 nm and 454 nm; MS: M-1 = 819.32 (calc 818.96); ddA4P-DDAO: UV λ A = 263 nm and 457 nm; MS: M-1 = 843.30 (calc 842.97); ddG4P-DDAO: UV λ A = 257 nm and 457 nm; MS: M-1 = 859.40 (calc 858.97).



ddT4P-DDAO

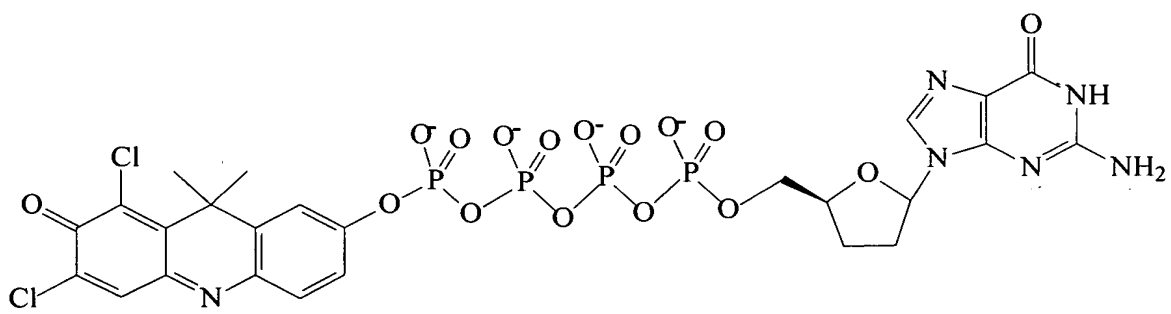


ddC4P-DDAO



ddA4P-DDAO

5



ddG4P-DDAO

10

Example 4

Preparation of ϵ -9H (1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)-dideoxythymidine-5'-pentaphosphate DDAO-ddT-pentaphosphate (ddT5P-DDAO)

5 A. Preparation of DDAO pyrophosphate

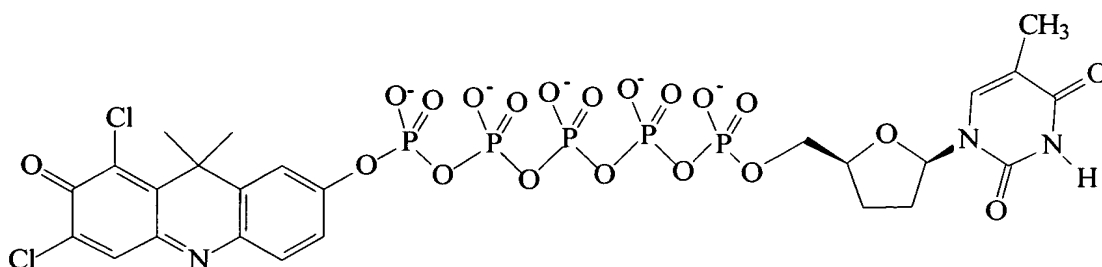
DDAO-phosphate diammonium salt (11.8 μ mol) was coevaporated with anhydrous DMF (3x 0.25 ml) and was dissolved in DMF (0.5 ml). To this carbonyldiimidazole (CDI, 9.6 mg, 5 eq) was added and the mixture was stirred at room temperature overnight. Excess CDI was destroyed by addition of MeOH (5 μ l) and stirring for 30 minutes. To the mixture tributylammoniumdihydrogen phosphate (10 eq., 236 ml of 0.5 M solution in DMF) was added and the mixture was stirred at room temperature for 4 days. Reaction mixture was concentrated on rotavap. Residue was purified on HiPrep 16.10 Q XL column using 0-100% B using 0.1M TEAB/acetonitrile (3:1) as buffer A and 1 M TEAB/acetonitrile (3:1) as buffer B. Main peak (HPLC purity 98%) was collected, concentrated and coevaporated with methanol (2x). Residue was dissolved in 1 ml water to give 5.9 mM solution. UV/VIS λ_{max} = 456 nm.

20 B. Preparation of ddT5P-DDAO

ddTTP (100 μ l of 47.5 mM solution in water) was coevaporated with anhydrous DMF (2x1 ml). To this DCC (5 eq., 4.9 mg) was added and mixture was coevaporated with DMF (1x1 ml). Residue was taken in anhydrous DMF (0.5 ml) and stirred at room temperature for 3 hours. To this 1.03 eq of DDAO pyrophosphate, separately coevaporated with anhydrous DMF (2x1 ml) was added as a DMF solution. Mixture was concentrated to dryness and then taken in 200 μ l anhydrous DMF. Mixture was heated at 38°C for 2 days. Reaction mixture was concentrated, diluted with water, filtered and purified on HiTrap 5 ml ion exchange column using 0-100% A-B using a two step gradient. Solvent A = 0.1M TEAB/acetonitrile (3:1) and solvent B = 1M TEAB/acetonitrile (3:1). Fraction 12 x 13 which contained majority of product were combined, concentrated and coevaporated with methanol (2x). Residue was repurified on Xterra RP C-18 30-100 mm column using 0.30% acetonitrile in 0.1M TEAB in 5 column and 30-50% acetonitrile in 2 column volumes, flow rate 10

ml/min. Fraction containing pure product was concentrated and coevaporated with methanol (2x) and water (1x). HPLC purity at 455 nm > 99%. UV/VIS = 268 nm and 455 nm. MS: M-1 = 914.03 (calc 913.93).

- 5 The DDAO dye attached to the gamma phosphate of these polyphosphates is fluorescent with an excitation maximum of 455 nm and an emission maximum of about 608 nm. Upon hydrolysis of the phosphate ester to release the free dye, the spectrum changes with excitation maximum of about 645 nm and emission maximum of about 659 nm. The change is readily detected by simple fluorescence
- 10 measurements or color change.



ddT5P-DDAO

- It is noted that similar nucleotide compounds with dyes or other detectable
- 15 moieties attached to the terminal phosphate could also be made using similar methods to those described in Examples 1-4 above. These include ribonucleotides, deoxyribonucleotides, nucleoside-tetraphosphates, nucleotides with any of the naturally-occurring bases (adenine, guanine, cytosine, thymine, hypoxanthine and uracil) as well as modified bases or modified sugars.

20

Example 5

Preparation of δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)- deoxythymidine-5'-tetraphosphate (dT4P-DDAO)

- 25 10 μ moles TTP TEA salt was evaporated to dryness. To the residue was added 40 μ moles tributylamine and 5 ml dry pyridine. The solution was re-evaporated to

dryness. After 2 coevaporations with 3ml dry dimethylformamide (DMF), residue was re-dissolved in 200 μ l dry DMF, flushed with argon and stoppered. Using a syringe, 50 μ moles (8 mg) carbonyldiimidazole (CDI) dissolved in 100 μ l dry DMF was added. The flask was stirred for 4 hr at ambient temperature.

5

While the above reaction was progressing, 35 mg (83 μ moles) DDAO phosphate and 166 μ moles tributylamine were dissolved in dry DMF. The DDAO phosphate was evaporated to dryness followed by 3 coevaporations with dry DMF. Residue was dissolved in 300 μ l dry DMF.

10

After the 4 hr reaction time, 3.2 μ l anhydrous methanol was added to the TTP-CDI reaction. The reaction was stirred 30 minutes. To this mixture, DDAO phosphate solution was added and mixture was stirred at ambient temperature for 18 hr. The reaction was checked by Reverse phase HPLC (Xterra 4.6x100 column, 0.1M TEAA/ acetonitrile). The reaction volume was reduced to 200 μ l by evaporation and the reaction was allowed to progress for 80 hr.

15

After 80 hr, the reaction was stopped by adding 15 ml 0.1 M TEAB. The diluted mixture was applied to a 19x100 Xterra RP column and eluted with an acetonitrile gradient in 0.1M TEAB. The fractions containing pure DDAO T4P were evaporated to dryness and coevaporated twice with ethanol. The residue was reconstituted with MilliQ water. Yield: 1.10 μ mole, 11%; HPLC purity > 98% at 455 nm; MS: M-1 = 850.07 (calc. 849.95)

20

δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)- deoxyguanosine-5'-tetraphosphate (dG4P-DDAO), δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)- deoxycytidine-5'-tetraphosphate (dC4P-DDAO) and δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)- deoxyadenosine-5'-tetraphosphate (dA4P-DDAO) were prepared in a similar manner as described above except 3.5 equivalents of DDAO phosphate was used instead of 8.3 equivalents. After C18 purification, samples were purified on ion exchange using a Mono Q 10/10 column.

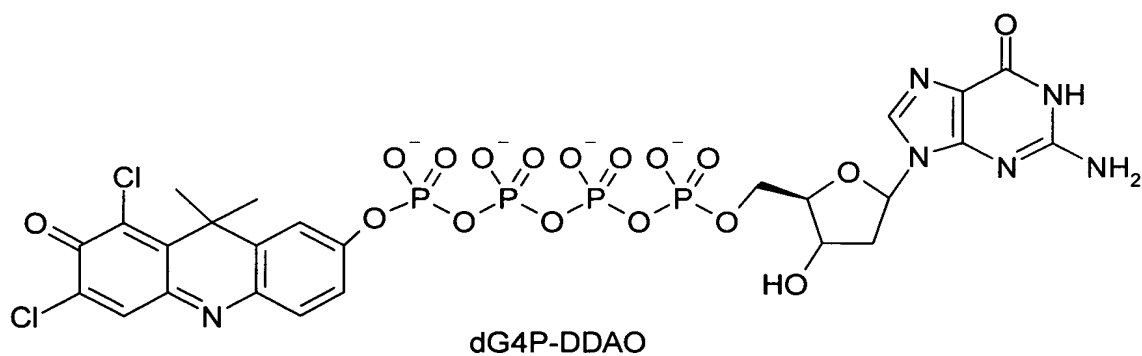
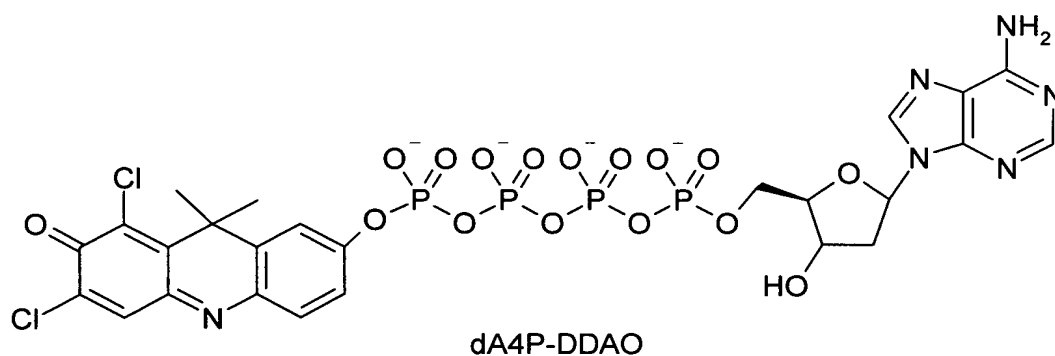
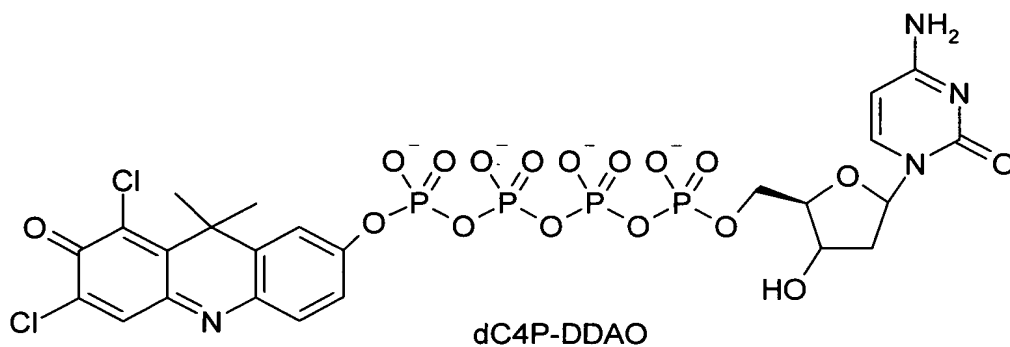
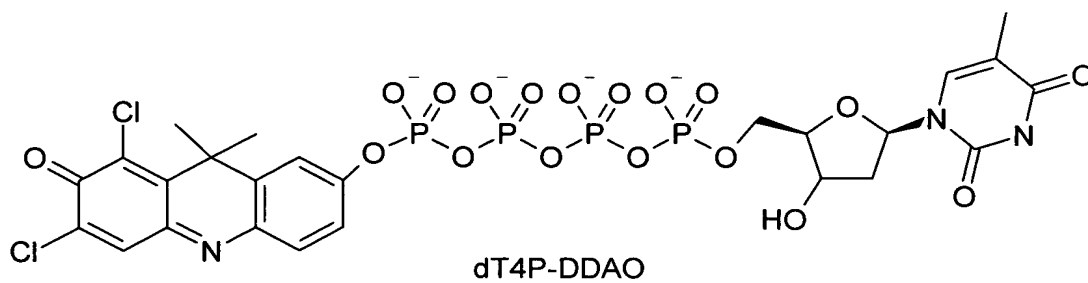
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30

δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)- deoxyguanosine-5'-
tetraphosphate (dG4P-DDAO): Yield 0.57 μ mol, 5.7%; HPLC purity 99% at 455 nm;
MS: M-1 = 875.03 (calc. 874.96).

5 δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)- deoxycytidine-5'-
tetraphosphate (dC4P-DDAO): Yield 0.24 μ mole, 2.4%; HPLC purity 99% at 455 nm;
MS: M-1 = 835.03 (calc. 834.95).

10 δ -9H(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)- deoxyadenosine-5'-
tetraphosphate (dA4P-DDAO): Yield 0.38 μ mole, 3.8%; HPLC purity 99% at 455
nm; MS: M-1 = 859.07 (calc. 858.97).



Examples 6, 7 and 8 below demonstrate that nucleotides having a dye derivative attached to the terminal phosphate may be effectively incorporated as substrates into a growing nucleic acid chain by a nucleic acid polymerase in a template-directed process for detection of a nucleic acid sequence.

5

Example 6

Nucleic Acid Sequence detection using polymerase incorporation of gamma phosphate-labeled ddGTP

10 Reactions were assembled at room temperature (23°C) using the dideoxynucleotide of Example (1). Reactions contained primer template combinations having a single oligonucleotide primer (represented by SEQ ID NO: 1) annealed to one of two different oligonucleotide templates with either a dC or a dT as the next template nucleotide adjacent the 3' terminus of the primer, corresponding to SEQ ID
15 NO: 2 and SEQ ID NO: 3, respectively.

Primer:

5'-GTT CTC GGC ATC ACC ATC CG-3' (SEQ ID NO: 1)

 Referring now to Figure 1, for template 1 (SEQ ID NO: 2) in the present
20 example, DNA polymerase would be expected to extend the primer with labeled ddGTP. Similarly, for template 2 (SEQ ID NO: 3) in Figure 1, DNA polymerase would be expected to extend the primer with ddATP, but not with labeled ddGTP.

Template #1:

5'-CAC CCT TAT CTG GTT GTC CAC GGA TGG TGA TGC CGA GAA C-3'
25 (SEQ ID NO: 2)

Template #2:

5'-CAC CCT TAT CTG GTT GTC TAC GGA TGG TGA TGC CGA GAA C-3'
(SEQ ID NO: 3)

30 Reaction conditions: A 70 µl reaction containing 25 mM Tris, pH 8.0, 5% glycerol 5 mM MgCl₂, 0.5 mM beta-mercaptoethanol, 0.01% tween-20, 0.25 units shrimp alkaline phosphatase, 100 nM primer annealed to template (the next template nucleotide is either dCMP or dTMP, as indicated), and 2 µM ddGTP-CF₃-Coumarin was assembled in a quartz fluorescence ultra-microcuvet in a LS-55 Luminescence

Spectrometer (Perkin Elmer), operated in time drive mode. Excitation and emission wavelengths are 390 nm and 500 nm respectively. Slit widths were 5 nm for excitation slits, 15 nm for emission slits. The reaction was initiated by the addition of 0.35 μ l (11 units) of a cloned DNA polymerase I genetically engineered to eliminate 3'-5' exonuclease activity, 5'-3' exonuclease activity and discrimination against dideoxynucleotides and 0.25 mM MnCl₂.

As shown in Figure 1, for reactions containing the gamma labeled ddGTP, dye emission was detected only with Primer: Template 1, where the next nucleotide in the template was a dC. Cleavage of the pyrophosphate product of phosphoryl transfer by shrimp alkaline phosphatase leads to a detectable change in the CF3-coumarin label which allows for the detection of the nucleic acid. No detectable dye emission was obtained with Primer: Template 2.

Example 7

Nucleic Acid Sequence detection using polymerase incorporation of gamma phosphate-labeled ddATP

Reactions were assembled at room temperature (23°C) using the dideoxynucleotide of Example (2). Reactions contained primer: template combinations having a single oligonucleotide primer (SEQ ID NO: 1) annealed to one of two different oligonucleotide templates with either a dC or a dT as the template nucleotide, adjacent to the 3' terminus of the primer, corresponding to SEQ ID NO: 2 and SEQ ID NO: 3, respectively.

Referring now to Figure 2, for template 2 (SEQ ID NO: 3) in the present example, DNA polymerase would be expected to extend the primer with labeled ddATP. Similarly, for template 1 (SEQ ID NO: 2) in Figure 2, DNA polymerase would be expected to extend the primer with ddGTP, but not with labeled ddATP.

Reaction conditions: A 70 μ l reaction containing 25 mM Tris, pH 8.0, 5% glycerol 5 mM MgCl₂, 0.5 mM beta-mercaptoethanol, 0.01% tween-20, 0.25 units shrimp alkaline phosphatase, 100 nM primer annealed to template, and 2 μ M ddATP-CN-Coumarin was assembled in a quartz fluorescence ultra-microcuvet in a LS-55

Luminescence Spectrometer (Perkin Elmer), operated in time drive mode. Excitation and emission wavelengths are 410 nm and 450 nm respectively. Slit widths were 5 nm for excitation slits, 15 nm for emission slits. The reaction was initiated by the addition of 0.35 μ l (11 units) of a cloned DNA polymerase I genetically engineered to eliminate 3'-5' exonuclease activity, 5'-3' exonuclease activity and discrimination against dideoxynucleotides and 0.25 mM MnCl₂.

As shown in Figure 2, for reactions containing the gamma labeled ddATP, dye emission was detected only for Primer : Template 2, where the next nucleotide in the template was a dT. Cleavage of the pyrophosphate product of phosphoryl transfer by shrimp alkaline phosphatase produces a detectable change in the CN-coumarin label that allows one to detect the nucleic acid. No detectable dye emission was obtained with Primer : Template 1.

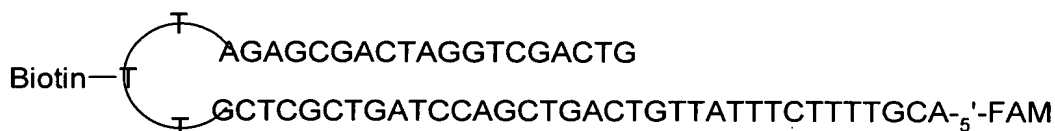
Example 8

Sequencing of a synthetic target on solid support

A. Immobilization of primer-target complex:

A suspension of dynabeads (M-270 streptavidin coated magnetic beads, 200 μ l of 10 mg/ml) was taken in an eppendorf and placed in a magnetic holder. Supernatant was removed with pipette and the tube was removed from the magnetic holder. Beads were resuspended in 1xPBS containing 0.01% Tween-20 (450 μ l) and tube was replaced in the holder. After removal of supernatant, the process was repeated with 1xPBS (450 μ l). Beads were resuspended in 1xPBS-Tween buffer (190 μ l) and a labeled oligonucleotide (a biotinylated template-primer of sequence shown below, e.g. SEQ ID NO: 4, labeled with fluorescein on the 5'-end, 10 μ l of 50 μ M aqueous solution). Mixture was incubated at 37°C for 30 minutes in a heated block with shaking. Supernatant was removed and beads were washed with 1xPBS-Tween (1 ml) and 1xPBS (1 ml). Beads were resuspended in 1 ml PBS and stored in a refrigerator. For oligo loading analysis, 100 μ l of the bead suspension was taken in an eppendorf and placed in the magnetic holder. After removal of supernatant, concentrated ammonium hydroxide (100 μ l) was added. Tube was closed and the suspension was incubated at 65°C for 10 minutes in a heating block with shaking to

release the oligo. Tube was placed in the magnetic holder and supernatant was removed. It was adjusted to 100 µl with 1xPBS and placed in a microtitre plate. In separate wells, standards containing 25.000 pmol, 12.500 µmol, 6.250 µmol, 3.125 µmol and 1.562 µmol of labeled oligonucleotide per 100 µl of 1xPBS buffer were placed and plate was scanned on a TECAN Ultra scanner for fluorescence emission. Loading was determined by fitting the data to a straight line and was found to be 13.94 pmol per 100 µl of the final bead suspension corresponding to 20 µl of original bead suspension.



10 SEQ ID NO: 4

B. Sequence determination:

Following buffer and nucleotide solutions were prepared: 5 ml of 25 mM Hepes pH 8.2, 5 mM MgCl₂, 0.5 mM MnCl₂, 0.01% Tween, 0.125u/µl TSI polymerase and 0.0026u/µl of Shrimp Alkaline Phosphatase (SAP). 25 µl of 100 µM solutions of each dN4P-(4-Me-coumarin) (N = G, T or C) was separately mixed with 475 µl of above buffer. A 50 µl of a 100 µM solution of dA4P-(4-Me-coumarin) was mixed with 950 µl of the above buffer.

Magnetic beads preloaded with oligo (10 µl of the loaded bead suspension with 1.39 pmol of oligo) were washed with the above buffer (2x 50 µl) using the magnetic separator. To the beads, 50 µl of a single nucleotide solution was added following the order GCTA-GATC-GCTA-GCAT-GTA-AG-GA-A-C-G. Thus, in the first cycle dG4P-(4-Me-coumarin) was added, in the 2nd cycle dC4P-(4-Methyl-coumarin) was added and so on. After addition of each nucleotide, beads were incubated at 37°C for 5 min at a shaker speed of 1400, separated on the separator and supernatant was placed in a marked well. Also prior to adding the next nucleotide, beads were washed with water (2x 50 µl) and above buffer (1x 50 µl). Each washing was placed in a separately marked well for reading. In separate wells 50 µl of each of

the above nucleotide solutions were placed to determine background fluorescence. In yet another set of wells, nucleotide solutions treated with Snake Venom Phosphodiesterase (known to cleave these nucleotides to generate dye phosphate) and diluted 10x with above buffer containing phosphatase were placed as standards to
5 determine total possible signal. A ratio of signal generated per nucleotide addition to the total possible signal can be used for quantification purposes. Plate was read at different intervals and at the end of experiment on a TECAN ultra scanner. Samples were excited at 360 nm and read at 465 nm. Raw fluorescence count (from supernatant and washings) after addition of each nucleotide mix was corrected by
10 subtracting the background present in that nucleotide solution. Expected values at each addition were calculated by multiplying the number of bases expected to be incorporated based on the sequence of template with the fluorescence count per nucleotide incorporated in the previous incorporation event.

15 Those skilled in the art, having the benefit of the teachings of the present invention as set forth above, can effect numerous modifications thereto. These modifications are to be construed as being encompassed within the scope of the present invention as set forth in the appended claims.